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Parasitism by protozoan *Ichthyophthirius multifiliis* enhanced invasion of *Aeromonas hydrophila* in tissues of channel catfish

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ABSTRACT

Protozoan *Ichthyophthirius multifiliis* Fouquet (Ich) and bacterium *Aeromonas hydrophila* are two common pathogens of cultured fish, which cause high fish mortality. Currently there is no information available for the effect of parasitism by Ich on survival of channel catfish and invasion of *A. hydrophila* in fish tissues following exposure to *A. hydrophila*. A trial was conducted in this study to: (1) determine whether *A. hydrophila* increased fish mortality in Ich-parasitized channel catfish; and (2) compare the bacterial quantity in different tissues between non-parasitized and Ich-parasitized catfish by real-time polymerase chain reaction (qPCR). The results demonstrated that the Ich-parasitized catfish showed significantly ($P < 0.05$) higher mortality (80%) when exposed to *A. hydrophila* by immersion than non-parasitized fish (22%). Low mortality was observed in catfish exposed to Ich alone (35%) or *A. hydrophila* alone (22%). *A. hydrophila* in fish tissues were quantified by qPCR using a pair of gene-specific primers and reported as genome equivalents per mg of tissue (GEs/mg). Skin, gill, kidney, liver and spleen in Ich-parasitized fish showed significantly higher load of *A. hydrophila* (9400–188,300 GEs/mg) than non-parasitized fish (4700–42,100 GEs/mg) after exposure to *A. hydrophila*. This study provides evidence that parasite infections enhance bacterial invasion and cause high fish mortality.

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1. Introduction

The ciliated protozoan *Ichthyophthirius multifiliis* Fouquet (Ich) is a common parasite of freshwater fish (Paperna, 1972; Jessop, 1995; Dickerson and Dawe, 1995; Traxler et al., 1998; Matthews, 2005). Ichthyophthiriasis frequently causes mass kills of cultured fish and leads to high economic losses to aquaculture. Ich infection can occur at any growth stages of fish, from day-old fry, fingerling, food size to brood fish. Some studies demonstrated enhanced bacterial invasion where damage caused by parasites serve as portals of entry (Cusack and Cone, 1986; Busch et al., 2003; Evans et al., 2007; Xu et al., 2007, 2009), thus causing increased mortality.

Motile *Aeromonas* septicemia (MAS) is a common fish bacterial disease and caused by *Aeromonas hydrophila*, *Aeromonas sobria* and *Aeromonas caviae* (Austin and Austin, 1987). In the United States, MAS primarily causes disease in cultured warm water fishes: channel catfish (*Ictalurus punctatus*), tilapia (*Oreochromis* spp.), carp (*Cyprinus carpio*), striped bass (*Morone saxatilis*), largemouth bass (*Micropterus salmoides*), bait fishes and others (Cipriano et al., 1984; Plumb, 1994). *A. hydrophila* also affects a variety of cool and cold-water fish species (Cipriano et al., 1984). No fish species is known to be totally resistant to *A. hydrophila* (Plumb, 1994). Most MAS epizootics in warm water fishes in the Southeastern United States generally occur in spring and early summer (Meyer, 1970; Thune and Plumb, 1982). The synonyms of MAS include hemorrhagic septicemia, infectious dropsy, dermal ulceration, tail or fin rot, ocular ulcerations, and rubella (Cipriano et al., 1984; Plumb, 1994). A fatal septicemia may occur rapidly in acute

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form, causing fish mortality before clinical signs develop (Cipriano et al., 1984).

Usually considered a secondary pathogen, *A. hydrophila* can also act as a primary pathogen in some environments, causing outbreaks in fish farms with high mortality and resulting in heavy economic losses worldwide (Plumb, 1994; Nielsen et al., 2001). Between June and October of 2009, a disease outbreak occurred in West Alabama, causing an estimated loss of more than 3 million pounds of food size channel catfish (Pridgeon and Klesius, 2011). The AL09-71 isolate was isolated from an infected channel catfish in August, 2009 from a farm located in AL. The disease outbreak caused a total loss of 15,000 lbs of channel catfish to that farm (Pridgeon and Klesius, 2011). The AL09-71 isolate showed LD_{50} value of 4.8×10^4 colony forming unit per fish to channel catfish by intraperitoneal injection (Pridgeon and Klesius, 2011).

Currently there is no information available on the effect of prior infection by Ich on the virulence of *A. hydrophila* to channel catfish. The objective of this study was to: (1) determine whether prior Ich parasitism would increase the mortality caused by *A. hydrophila* to channel catfish; and (2) determine whether prior Ich parasitism would increase the amount of *A. hydrophila* in different tissues of channel catfish.

2. Materials and methods

2.1. Fish and parasite

Channel catfish (industry pool strain) were obtained from disease-free stock from the USDA-ARS Catfish Genetic Research Unit, Stoneville, MS and reared to the experimental size in indoor tanks at the USDA, Aquatic Animal Health Research Laboratory, Auburn, AL. *I. multifiliis* (ARS 10-1 strain) was originally isolated from infected pet fish obtained from a local pet shop and maintained by serial transmission on channel catfish held in 50-l glass aquaria as previously described (Xu et al., 2004).

Fish infected with maturing trophonts were anesthetized with 150 mg/l tricaine methanefulfonate (MS-222), rinsed in tank water and the skin was gently scraped to dislodge the parasites. Isolated trophonts were placed in a tank with 20-l water and incubated at 22–24 °C. Theronts for infection trials were enumerated with the aid of a Sedgewick-Rafter cell.

2.2. Water quality

During trials, dissolved oxygen (DO) and temperature in tanks were measured daily using a YSI 85 oxygen meter (Yellow Spring Instrument, Yellow Springs, OH). The pH, hardness, ammonia and nitrite were determined using Hach CEL/890 Advanced Portable Laboratory (Loveland, CO).

2.3. Bacterial isolation

An isolate of *A. hydrophila* (AL09-71) was obtained from diseased catfish and cultured on blood agar plates (Difco tryptic soy agar with 5%, v/v, defibrinated sheep blood) as described by Pridgeon and Klesius (2011). The isolate of *A. hydrophila* was then characterized biochemically by standard biochemical tests as described in Bergey's Manual of Determinative Bacteriology (Holt et al., 1994) and API 20E bacterial identification test strip manufactured by Biomérieux, Inc. Single colonies picked from an agar plate were transferred to tryptic soy broth (TSB), cultured at 28 °C in a shaker for 24 h and used to challenge fish. The optical densities (OD) of 1.0 of the bacterial cultures were measured at 540 nm using SmartSpec 3000 spectrophotomer (Bio-Rad, Hercules, CA) and the concentration (colony forming units per milliliter CFU/ml) of *A. hydrophila* was determined through serial 1:10 dilution using standard plate-count. Dead or moribund fish were removed twice daily during the infectious trial and bacterial samples aseptically obtained from liver and kidney were streaked onto blood agar plates. The biochemical tests described above were used to confirm the identity of *A. hydrophila* from the dead fish.

2.4. Experimental design and challenge procedure

A total 220 channel catfish, which ranged from 11.2 ± 0.5 cm (mean ± SEM) in total length and 9.6 ± 1.3 g in body weight ($N = 10$) were used in this trial. All fish treatment protocols were approved by Institutional Animal Care and Use Committee at the Aquatic Animal Health Research Laboratory. Ten catfish were examined and cultured to verify pathogen free status of parasite and bacteria prior to the trial. All fish were negative for parasites and fish pathogenic bacteria.

Fish were distributed to eight 57-l tanks with four tanks exposed to Ich and four tanks as no parasite control. Water was lowered to 10 l in each tank prior to Ich theront exposure. For fish infected with Ich, Ich theronts were added to each tank at 20,000 theronts per fish and the fish were exposed to theronts for 1 h with aeration. The fish in the remaining tanks were not exposed to Ich theronts but kept in 10 l water for 1 h with aeration. Water flow (0.5 l/min) was resumed after 1 h and the fish were monitored for Ich infection in those aquaria. When fish showed visible "white spots" 5 d post theront exposure, 5 fish from Ich infection and 5 fish from control (non-infected) tanks were sampled to verify the *A. hydrophila* free status in different tissues with qPCR. The skin, gill, liver, kidney, and spleen were sampled using aseptic technique. Then parasite infections on skin/fins were determined for 5 fish from each Ich infection tank (a total of 20 infected fish). Parasite infection level of each fish was assessed while the fish was kept in a 2-l beaker as none, light (<50 trophonts/fish), medium (50–100 trophonts/fish) and heavy infection (>100 trophonts/fish) (Xu et al., 2004). After parasite evaluation, all fish were distributed into 20 tanks with 10 fish/tank that received the treatments listed in Table 1. For fish challenged with *A. hydrophila*, 100 ml bacterial suspension (3×10^9 CFU/ml) was added to a 2-l beaker filled with 1 l tank water and 10 fish were exposed to *A. hydrophila* with aeration for 1 h. For fish without *A. hydrophila* challenge, the same amount of TSB broth was added to each beaker. After challenge, the fish from each beaker were moved to a 57-l aquarium with flowing water

Table 1
Replicates and fish number in each treatment in the infection trial. For groups 1 and 3, four tanks were monitored for fish mortality and two tanks sampled to quantify *A. hydrophila* in different tissues with qPCR.

Group	Treatment	Replicate	Fish number
1	Infected by Ich and *A. hydrophila*	6	60
2	Infected by Ich only	4	40
3	Infected by *A. hydrophila* only	6	60
4	TSB immersion only	4	40

at 0.5 l/min with aeration. Four tanks of fish in groups 1–4 were monitored for mortality daily after *A. hydrophila* challenge. The mortality of fish was recorded and dead fish were examined for parasite and *A. hydrophila* infection twice daily for 2 wk. The remaining 2 tanks in the group 1 or 3 were sampled to quantify *A. hydrophila* in different tissues with qPCR. The skin, gill, liver, kidney, and spleen from two fish in each tank (4 fish per group) were sampled using aseptic technique at hour 5, day 1, day 2, and day 7 post challenge with *A. hydrophila* after fish were anesthetized with 300 mg/l MS-222.

2.5. Genomic DNA isolation from bacterial cell culture and standard curve

A pure culture of *A. hydrophila* (AL09-71) was incubated in TSB broth at 28 °C in a shaker overnight and adjusted spectrophotometrically to OD 1.0 at 540 nm, corresponding to 2.4×10^{10} CFU/ml by the standard plate-count method. Four tubes of 1 ml bacterial culture solution were centrifuged at $9000 \times g$ in Microfuge centrifuge (Beckman Coulter) for 5 min and the supernatant discarded. The genomic DNA (gDNA) from bacterial pellets was extracted and purified using DNeasy tissue kit following the bacterial protocol in the manufacturer's instructions (Qiagen). DNA yield and purity were determined spectrophotometrically using Nanodrop ND-1000. The purified gDNA was stored at −20 °C until use. The DNA from the bacteria used in specificity test was isolated using the same protocol as for *A. hydrophila*. For standards, the gDNA of *A. hydrophila* was made 10-fold serial dilutions from 5 ng/μl to 5 fg/μl with sterile water or tissue extracts (skin, gills, liver, kidney, and spleen) prepared as described below.

2.6. Genomic DNA isolation from fish tissues

The tissues used to quantify *A. hydrophila* with qPCR were preserved at −20 °C for DNA extraction. Twenty mg of each fish tissue were weighed and macerated with sterilized Kontes disposable pestles in a microcentrifuge tube. Total genomic DNA of *A. hydrophila* in fish tissues was extracted by the DNeasy Tissue kit (Qiagen, USA) and eluted into 200 μl water according to the manufacturer's instructions. If the sample did not weigh exactly 20 mg, the extracted DNA was eluted into a volume of water equal to 10 μl water per mg tissue. DNA yield and purity were determined spectrophotometrically using Nanodrop ND-1000 (NanoDrop Technologies, Wilmington, DE, USA). The purified and concentrated gDNA was stored at −20 °C until use. The extracted DNA in fish tissue was further diluted to 1:5 for qPCR assay and 1-μl of eluted/diluted sample was used as template in qPCR. The DNA concentration in fish tissue was determined using the standard curve (threshold cycle (C_t) values vs. DNA concentration of *A. hydrophila*). Since 1-μl of eluted/diluted sample was run in qPCR, the amount of bacterial DNA in each mg of tissue was equal to bacterial DNA concentration (pg/μl) × eluted volume × dilution factor/tissue weight in mg. The bacterial DNA in each mg of tissue was further calculated as genome equivalents per mg of tissue (GEs/mg) based on the 4.7 mbp genome size of *A. hydrophila* (Seshadri et al., 2006) using a conversion factor 1 pg = 978 mbp (Doležel et al., 2003).

2.7. Primer design and real-time PCR

The PCR primers were designed by using Primer3 program (http://frodo.wi.mit.edu/primer3/). A pair of gene-specific primers, forward Aero2F 5′-CGCCAGCTGGTCAAGACTGT-3′ and reverse Aero2R 5′-CCAGTTGGTGGCTGTGTCGT-3′, was designed to target a nucleotide region spanning 1180–1281 bp of the hole-forming preprotoxin aerolysin gene of *A. hydrophila* (GenBank accession number: M16495.1). The 102 bp amplicon showed no homology to other published sequences of bacteria in freshwater (bacteria other than *A. hydrophila*) in the GenBank database.

One-step quantitative real-time PCR (qPCR) analysis was performed on an Applied Biosystems 7500 Real-Time PCR System (ABI, Foster City, CA) using Platinum® SYBR® Green qPCR SuperMix-UDG (Invitrogen, Carlsbad, CA). The qPCR was performed in a final volume of 12.5 μl as described by Pridgeon et al. (2010) and the qPCR mixture consisted of 1 μl of gDNA from tissue samples, 0.5 μl of 5 mM forward primer, 0.5 μl of 5 mM reverse primer and 10.5 μl of 1x SYBR Green SuperMix. Reactions were analyzed with an Applied Biosystems 7500 under the following conditions: 50 °C for 2 min, 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. All qPCR was run in duplicate for each gDNA sample and four fish gDNA samples from each treatment group were analyzed. If the standard deviation in duplicated wells was above 0.30, indicating a variation in assay precision (True et al., 2009), the sample was re-assayed. The same tissues collected from different treated fish and from different sampling times were quantified for gDNA of *A. hydrophila* in the same 96 well plate to avoid plate variation.

2.8. Sensitivity and specificity of the qPCR assay

The detection limit was evaluated from 5 ng/μl to 5 fg/μl of the gDNA of *A. hydrophila* and the reliable endpoint was determined by examining the standard deviation of the threshold cycle (C_t) values of four replicate wells. Standard deviations above 0.30 were used to identify gDNA concentrations in which replicates no longer conformed to assay precision as recommended by Applied Biosystems, Inc. (True et al., 2009). Specificity of the qPCR was determined by performing the assay on DNA extracted from cultures of bacteria listed in Table 2. Most bacteria in the list were originally isolated from diseased fish, identified

Table 2
Bacterial species used to determine the specificity of the qPCR.

Isolate	Species	Origin	Source
AL09-71	*Aeromonas hydrophila*	Alabama	Channel catfish
AH10-12-19A	*A. hydrophila*	Alabama	Channel catfish
ATCC 7966	*A. hydrophila*	ATCC	Tin of milk with a fishy odor
AH98-69A	*A. hydrophila*	Alabama	Channel catfish
AL98-C1B	*A. hydrophila*	Alabama	Nile tilapia
ATCC-33658	*A. salmonicida*	ATCC	Atlantic salmon, *Salmo salar*
ATCC 15468	*A. caviae*	ATCC	Guinea pig, *Cavia pocellus*
ATCC 43979	*A. sobria*	ATCC	Fish (species unknown)
EILO (Eic2)	*Edwardsiella ictaluri*	Thailand	*Clarias batrachus* L.
Eta8	*E. tarda*	Alabama	Nile tilapia
FC-1	*Flavobacterium columnare*	Alabama	Channel catfish
Sin17	*Streptococcus iniae*	Hawaii	Nile tilapia
Sag10	S. agalactiae	Idaho	*Oreochromis* spp.
ATCC 27853	*Pseudomonas aeruginosa*	ATCC	Blood culture

into species using standard methods and maintained at the USDA Aquatic Animal Health Research Unit, Auburn, AL (Panangala et al., 2007; Pridgeon and Klesius, 2011).

The C_t values and baseline settings were determined by automatic analysis settings. The levels of *A. hydrophila* aerolysin gene were determined by subtracting the C_t value of the sample by that of control (fish without exposure to *A. hydrophila*) using the formula $\Delta C_t = C_t$ (control) $- C_t$ (sample). The relative expression levels of aerolysin gene in fish exposed to both Ich and *A. hydrophila* (Ich–AH) were compared to that in fish without parasites but exposed to *A. hydrophila* (noP–AH) using the formula $E^{\Delta\Delta C_t}$, where E is the corresponding qPCR efficiency, $\Delta\Delta C_t = \Delta C_t$ (noP–AH) $- \Delta C_t$ (Ich–AH) as described by Pfaffl (2001) and Pridgeon et al. (2010). The qPCR amplification efficiency was calculated according to the equation $E = 10^{-1/\text{slope}} - 1$ (Bustin et al., 2009).

2.9. Statistical analysis

All data were analyzed with SAS software (SAS Institute, 1989). Median days to death (MDD) were calculated by Lifetest procedure (Kaplan–Meier method). Mortalities, MDD of fish, and the levels of *A. hydrophila* DNA in tissues from different treatment groups were compared with Duncan multiple range tests. *P*-values of 0.05 or less were considered statistically significant.

3. Results

3.1. Water quality

During the trial, the mean ± MSE of DO was 6.7 ± 0.3 mg/l, temperature was 24.6 ± 0.3 °C, pH was 7.2 ± 0.2, ammonia was 0.45 ± 0.2 mg/l, and hardness was 80.2 ± 4.8 mg/l. Nitrite concentrations were below the detection limit.

3.2. Effect of Ich infection on mortality of catfish after exposure to A. hydrophila

Channel catfish infected by Ich showed visible "spots" prior to *A. hydrophila* exposure. Among these infected fish, 47% fish showed a moderate infection (50–100 trophonts/fish) and 53% fish showed a heavy infection (>100 trophonts/fish). No Ich trophonts were observed on fish without exposure to theronts.

Parasitized catfish showed significantly ($P < 0.05$) higher mortality (80.0%) than non-parasitized fish (22.5%) after exposure to *A. hydrophila* by immersion. The co-infected fish (80%) also had a significantly higher mortality than fish infected by Ich alone (35%). All fish survived in the group without exposure to Ich and *A. hydrophila* (Table 3). The median days to death were 2.7 ± 1.8 days and 3.3 ± 0.6 days, respectively for parasitized fish and non-parasitized fish following challenge with *A. hydrophila*.

Dead fish were sampled to isolate *A. hydrophila* for 14 d post challenge. Almost all sampled fish were positive (≥94.4%) for *A. hydrophila* in groups of fish challenged with *A. hydrophila* (Table 3). No *A. hydrophila* was isolated from fish infected by Ich alone without exposure to *A. hydrophila*.

3.3. Sensitivity, specificity and efficiency of the real-time PCR

The real-time PCR detected 10-fold serially diluted samples containing 5 fg to 5 ng of *A. hydrophila* DNA. However, the standard deviation of the C_t values in replicate wells of 5 fg samples was above 0.30 so the precision for 5 fg was not reliable. In this study, we included 6 dilutions of DNA from 50 fg to 5 ng in the standard curve to quantify DNA. These DNA dilutions were equivalent to 1.04×10^1–1.04×10^6 genomes of *A. hydrophila* (Fig. 1).

Using the specific qPCR primers targeting the aerolysin gene of *A. hydrophila*, amplified products were detected in *A. hydrophila* isolates (AL09-71, AH10-12-19A, ATCC-7966, AH98-69A and AL98-C1b) in qPCR reactions. A band showing the 102 bp amplified product was observed in the 5 isolates of *A. hydrophila* tested and *Aeromonas salmonicida* (ATCC-33658) by qPCR and gel electrophoresis (Fig. 2). No bands were observed in isolates of *A. caviae* (ATCC-15468), *A. sobria* (ATCC-43979), *Edwardsiella ictaluri* (EILO), *Edwardsiella tarda* (Eta8), *Flavobacterium columnare* (FC-1), *Streptococcus iniae* (Sin17), *S. agalactiae* (Sag10) and *Pseudomonas aeruginosa* (ATCC-27853).

The qPCR amplification efficiency was determined for each tissue. The amplification efficiencies ranged from 0.90,

Table 3
Cumulative mortality of channel catfish infected by parasite *Ichthyophthirius multifiliis* (Ich) and bacterium *Aeromonas hydrophila*. Within a given column, means followed by different letters are statistically different ($P < 0.05$).

Parasite	Bacteria	Mortality[a] (%)	Fish sampled for *A. hydrophila* (Ich)	Positive to[b] *A. hydrophila* (Ich)	% positive to *A. hydrophila* (Ich)
I. multifiliis	*A. hydrophila*	80.0 ± 8.2a	18 (17)	17 (17)	94.4 (100)
I. multifiliis	TSB broth	35.0 ± 15.5b	10 (10)	0 (10)	0 (100)
No parasite	*A. hydrophila*	22.5 ± 11.0c	8 (8)	8 (0)	100 (0)
No parasite	TSB broth	0 ± 0d	0 (0)	0 (0)	0 (0)

[a] Fish mortality (±MSE) was the mean mortality of 40 fish from 4 tanks and observed for 15 days post challenge with *A. hydrophila*.
[b] Fresh dead or moribund fish were sampled to verify bacterial or parasite infection. Number of fish sampled, number of fish positive and percentage of fish positive to *A. hydrophila* were listed in the last 3 columns of the table. The same values for Ich are shown in parentheses.

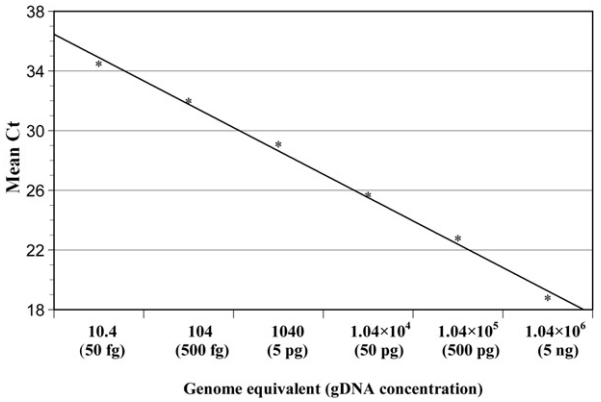

Fig. 1. Standard curve for quantifying *Aeromonas hydrophila* in skin of channel catfish. The nucleic acid extracted from pure culture of *A. hydrophila* was made 10-fold series dilution and added to skin extract from 5 ng to 50 fg per microliter, which were equivalent to $1.04 \times 10^6 - 10.4$ genomes of *A. hydrophila*. The threshold cycle (C_t) value is defined as the cycle in which fluorescence is first measured. The C_t values (*y*-axis) are plotted against the $\log_{10}$ dilution series (*x*-axis). The standard curve revealed a linear correlation between C_t values and log amount of nucleic acid ($Y = -3.3321X + 21.994$, $R^2 = 0.9925$).

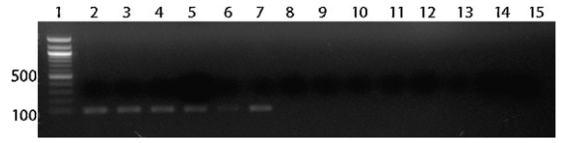

Fig. 2. Gel electrophoresis showed a 102 bp amplified products of aerolysin gene of *Aeromonas hydrophila*. Lane 1: 100 bp DNA ladder. Lanes 2–6: *A. hydrophila*: 2, AL09-71; 3, AH10-12-19A; 4, ATCC-7966; 5, AH98-69A; 6, AL98-C1B. Lane 7: *A. salmonicida* (ATCC-33658). Lane 8: *A. caviae* (ATCC-15468). Lane 9: *A. sobria* (ATCC-43979). Lane 10: *Edwardsiella ictaluri* (EILO). Lane 11: *E. tarda* (Eta8). Lane 12: *Flavobacterium columnare* (FC-1). Lane 13: *Streptococcus iniae* (Sin17). Lane 14: *S. agalactiae* (Sg10). Lane 15: *Pseudomonas aeruginosa* (ATCC-27853).

0.95, 0.99, 0.99, to 1.03 for spleen, kidney, liver, gill and skin, indicating a high degree of efficiency in qPCR.

3.4. Amount of *A. hydrophila* in skin and gills of fish

A. hydrophila in fish tissues were quantified by qPCR and reported as genome equivalents per mg of tissue (GEs/mg). No *A. hydrophila* was detected in parasitized fish or non-parasitized fish in all tissues prior to exposure to *A. hydrophila*. The parasitized fish exposed to *A. hydrophila* showed 10,800–14,400 GEs/mg in skin, significantly higher than non-parasitized fish (4700–6300 GEs/mg) from hour 5 to day 2 post *A. hydrophila* exposure (Table 4). The amounts of *A. hydrophila* in skin of fish infected by Ich followed by *A. hydrophila* infection were 2.3, 2.5, and 1.8 fold more than non-parasitized fish when sampled at hour 5, 1 d and 2 d post bacterial exposure, respectively.

The bacterial loads were significantly higher in gill of parasitized fish (27,200–81,000 GEs/mg) than non-parasitized fish (16,000–20,600 GEs/mg) after exposure to *A. hydrophila* (Table 4). The amounts of *A. hydrophila* in gills of parasitized fish were 2.3, 2.5 and 1.8 fold greater than non-parasitized fish when sampled at 5 h, 1 d and 2 d post *A. hydrophila* exposure, respectively.

3.5. *A. hydrophila* in the internal organs of channel catfish

The kidney of parasitized catfish showed significantly ($P < 0.05$) higher numbers of bacteria

Table 4
Comparison of genome equivalents of *A. hydrophila* (±MSE) per mg of tissues (GEs/mg) from channel catfish between coinfection by *Ichthyophthirius multifiliis* and *A. hydrophila* (Ich–*A. hydrophila*) and single infection by *A. hydrophila* (*A. hydrophila*) at different time post bacterial exposure. Within a tissue, means followed by different letters are statistically different ($P < 0.05$).

Fish group	Time	Genome equivalents of *A. hydrophila* per mg of tissues (x 1000)				
		Skin	Gill	Kidney	Liver	Spleen
Ich–*A. hydrophila*	Hour 5	14.4 ± 2.4a	81.0 ± 19.3a	188.3 ± 10.2a	77.2 ± 3.3a	41.3 ± 12.6a
	Day 1	11.8 ± 1.8a	62.9 ± 11.5a	28.9 ± 2.9b	19.3 ± 3.1b	10.6 ± 1.8b
	Day 2	10.8 ± 2.2a	27.2 ± 13.0b	38.1 ± 12.0b	18.1 ± 5.5b	9.4 ± 2.4b
	Day 7	9.4 ± 2.8a	1.6 ± 0.8c	16.3 ± 7.2c	20.4 ± 7.2b	6.9 ± 0.3c
A. hydrophila	Hour 5	6.3 ± 0.4b	15.9 ± 5.4d	42.1 ± 14.1b	10.0 ± 3.3c	8.0 ± 2.5b
	Day 1	4.7 ± 0.4b	20.6 ± 5.8d	22.1 ± 2.9d	12.9 ± 3.1c	5.9 ± 1.0c
	Day 2	6.0 ± 0.9b	16.1 ± 3.2d	23.0 ± 8.5d	12.4 ± 3.8c	3.5 ± 0.6d
	Day 7	7.1 ± 1.8b	1.5 ± 1.1c	15.3 ± 6.8c	12.7 ± 4.3c	3.7 ± 0.3d

(28,900–188,300 GEs/mg) than non-parasitized catfish (22,100–42,100 GEs/mg) from 5 h to 2 d post exposure to *A. hydrophila* (Table 4). The amounts of *A. hydrophila* in kidney of parasitized fish were 4.5, 1.3 and 1.7 fold greater than non-parasitized fish when sampled at 5 h, 1 d and 2 d post *A. hydrophila* exposure, respectively. The parasitized catfish also demonstrated higher bacterial numbers in liver (18,100–77,200 GEs/mg) and spleen (6900–41,300 GEs/mg) than non-parasitized catfish (10,000–12,900 GEs/mg in liver and 3500–8000 GEs/mg in spleen) after exposure to *A. hydrophila*. The GEs of *A. hydrophila* in liver of parasitized catfish were 7.2, 1.5 and 1.5 fold higher than non-parasitized fish at 5 h, 1 d, and 2 d post exposure to *A. hydrophila*, respectively (Table 4). The spleen of parasitized fish also showed 5.2, 1.8 and 2.7 fold more *A. hydrophila* compared to that of non-parasitized fish at 5 h, 1 d and 2 d post exposure to *A. hydrophila*, respectively (Table 4).

4. Discussion

Previous studies demonstrated that Nile tilapia parasitized with *Gyrodactylus* or *I. multifiliis* suffered higher mortality than non-parasitized fish when exposed to *S. iniae* (Xu et al., 2007, 2009). *I. multifiliis* and *A. hydrophila* are two common pathogens of channel catfish. No study has been conducted to demonstrate that prior infection by Ich enhances the mortality following exposure to *A. hydrophila* in channel catfish. The current study demonstrated that infection by Ich significantly increased mortality of channel catfish following exposure to *A. hydrophila*. The results of this study and other studies (Cusack and Cone, 1986; Busch et al., 2003; Bandilla et al., 2006; Evans et al., 2007; Xu et al., 2007; Martins et al., 2011) strongly support the notion that prior parasite infections enhance the ability of the bacteria to cause fish mortality.

There are several possible roles of Ich parasitism in fish death when co-infected with *A. hydrophila*. The parasite: (1) directly damages fish skin/gills and causes fish death; (2) damages fish first line of defense and helps *A. hydrophila* gain entry into fish host; (3) causes stress and reduces fish immune protection thus increasing the ability of *A. hydrophila* to infect fish (Sitja-Bobadilla, 2008; Jørgensen and Buchmann, 2007). Fish mucus covering the epithelium provides protection against pathogens (Plumb, 1994). If the mucus layer is damaged, underlying epithelium is exposed to bacteria present in water and the skin and gills become portals of entry for bacteria (Plumb, 1994). Other studies have also suggested that parasite injuries are potential portals of entry for bacterial pathogens (Cusack and Cone, 1986; Busch et al., 2003; Bandilla et al., 2006; Evans et al., 2007). Jørgensen and Buchmann (2007) noted that Ich infections significantly increased the cortisol level in rainbow trout thus leading to an immuno-suppressed state. The decreased resistance in fish due to parasite stress could further increase fish susceptibility to the bacterial infection (Pickering and Pottinger, 1989; Plumb, 1994).

No study has quantified bacterial loads between parasitized fish and non-parasitized fish. In this study, qPCR and a pair of specific primers targeting the aerolysin gene of *A. hydrophila* were used to quantify bacterial load in tissues of fish. The results in this study demonstrated that numbers of bacteria entering fish tissues were greatly affected by Ich parasitism. Parasitized fish exhibited higher loads of *A. hydrophila* in skin, gill, kidney, liver and spleen than non-parasitized fish from 5 h to 2d post bacterial exposure. Most of fish died 1–3 days post *A. hydrophila* exposure. The median days to death were 2.7 days for parasitized fish following challenge with *A. hydrophila*. The bacterial loads in fish tissues were related well to fish mortality. The *A. hydrophila* (AL09-71) used in this study was a virulent strain which caused heavy loss in channel catfish from a farm located in AL in 2009 (Pridgeon and Klesius, 2011). In their virulence study, Pridgeon and Klesius (2011) found that *A. hydrophila* (AL09-71) isolate caused most infected fish to die within 24 h post IP injection of *A. hydrophila*.

One of difficulties of co-infection studies is managing concentration of pathogens used to infect fish. Either Ich or *A. hydrophila* alone can cause high fish mortality if the pathogen concentrations are too great. Alternatively, fish may show low or no mortality when pathogen concentrations are too low. In this study, we exposed fish to Ich theronts at 20,000 theronts per fish for 1 h. After fish showed visible "spots" on skin surface, fish were exposed to *A. hydrophila* at 3×10^{11} CFU/l by immersion for 1 h. The trial demonstrated that the fish co-infected with Ich and *A. hydrophila* showed the highest mortality, significantly higher than fish infected by Ich alone or infected by *A. hydrophila* alone.

Outbreaks of MAS are seasonal, with peaks generally occurring in the spring to early summer and in the fall when water temperatures are between 18 and 29 °C (Camus et al., 1998). The temperature ranges for MAS outbreaks overlap the optimum temperature window of Ich infection at 22–24 °C (Dickerson and Dawe, 1995; Matthews, 2005). There is no published information available on the effect of Ich infection on the virulence of *A. hydrophila* to channel catfish in fish farms. However, the two pathogens have been diagnosed in the same fish (Personal communication). The results from this study suggest that parasitism by Ich at fish farms could enhance infection by *A. hydrophila* and subsequently reduce fish survival. Prevention and treatment of parasite infection in fish will reduce direct damage and stress due to parasite infection ultimately reducing mortality resulting from secondary bacterial infections.

In summary, Ich-parasitized channel catfish showed significantly higher mortality when co-infected with *A. hydrophila*. This study utilized a qPCR method to determine bacterial load in fish tissues. Skin, gill, kidney, liver and spleen in Ich-parasitized fish showed significantly higher load of *A. hydrophila* (9400–188,300 GEs/mg) than non-parasitized fish (4700–42,100 GEs/mg) after exposure to *A. hydrophila*. This study provides evidence that parasitic infection enhanced bacterial invasion resulting in high fish mortality. The information can be used to develop comprehensive health management plans including co-infections to aid in minimizing fish loss.

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